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Effect of Numerous Amino Acids During in vitro Fertilization of Ovums and Epididymal Sperm in Iraqi Awassi Sheep

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Abstract

In present experiment, the effect of three amino acids, methionine, L-arginine, and D-alanine, on the process of producing embryos in the laboratory, was studied. A 100 mature ovaries of Awassi sheep slaughtered in the Al-Saadoun slaughterhouse in Nineveh Governorate/Iraq were used. The samples were transported using a refrigerated box after washing with physiological saline and antibiotics. The insemination took place in the IVF laboratory of Soran Hospital in Erbil Governorate from 1/9/2022 to 1/5/2023. An eighty mature eggs with very good characteristics were collected and divided into four groups (20 eggs per group) then specified amino acid was added to each group, three groups (amino acid per group) and the fourth group was left as a control group without addition. The ripening and inoculation process was carried out according to the protocol of the laboratory in which the experiment was conducted. Results of this experiment showed that the arginine group (0.4 Mm) recorded the highest fertilization rate obtained (87.5%) based on the evaluation of embryos when kept in a carbon dioxide incubator (CO2 incubator) after 120 hours of maturation and fertilization, followed by Methionine group (0.2 Mm), control group and finally alanine group (15 Mm), where the results were (85%), (66.6%), (0.0%) respectively depended up on embryo evaluation (Grade A embryo). It is concluded from the current study that the arginine was the best of the three amino acids in stimulating divisions of the egg and improving the percentage of embryos produced in the laboratory after incubation for 120 hours.

INTRODUCTION

Gametes are subjected during handling in the laboratory to many types of stress, and perhaps oxidative stress is the most prominent, as it causes damage or alteration of the genetic material of sperm and reduces the quality of eggs and has a decisive effect on the possibility of embryonic development even after implantation [1].

Oocytes can be exposed to high levels of free radicals during in vitro fertilization IVF due to the dead sperm surrounding them [2]. Thus, the oocytes and sperm are exposed together to free oxygen radicals (ROS), which affects their quality and ability to fertilize. fertilization [3].

The action of antioxidants and antioxidant supplements includes improving the quality of sheep embryos produced in the laboratory with modifications in key gene expressions [4]. Examples of antioxidants used in the processes of reducing free oxygen

radicals are amino acids, as they were used in almost all stages of production. Embryos in the laboratory [5], starting from incubation of sperm, maturation of eggs, fertilization, quality and development of embryos after fertilization, first embryonic divisions, formation of the ovum, blastocyst and embryonic vesicle [6] and an example of it is vitamin E, vitamin A, vitamin C, cysteine, alanine, arginine, methionine, (enzyme antioxidants) and melatonin.

Thus the purpose of present study was to investigate and comparison of adding three amino acids which were arginine, methionine and alanine during in vitro fertilization of Iraqi Awassi sheep ova and epididymal sperms collected from slaughterhouse.

Materials and methods

Animal ethical approval:

In this study, the objectives were accomplished through the utilization of samples obtained from a slaughterhouse. Therefore, it was determined that the need for research ethics committee permission was unnecessary.

Study structure:

The study were including adding of three amino acids which were Arginine (0.4 Mm), Methionine (0.2 Mm) and Alanine (15 Mm) into maturation, fertilization media during In vitro fertilization of Iraqi Awassi sheep ova and epididymal sperms, eighty grade A (very good quality according to presence of intact Zona Pellucida , clear cytoplasm, presence of surrounding granulosa cells) oocytes were collected and divided and placed into four petri dishes to contracted four groups (20 ova/dish), one amino acid for each petri dish and the last petri dish left without adding any amino acid as control group, then the all ovum processed under maturation and fertilization of approved sperms viability.

Site of Study:

The ovum (100 ovarian samples, total 80 grade A ova) and epididymal sperms (4 samples, total 8 testis) was collected and treated in the Artificial Insemination Laboratory, College of Veterinary Medicine, University of Mosul, Mosul city (the city's coordinates span between 2°36′ longitude and 43°7′ latitude) between1/9/2022 and 1/5/2023, Ewes genitalia and testis slaughtered at a local abattoir were collected within two hours of slaughter, the samples were washed with normal saline and antibiotic sand transferred in a refrigerated box. In vitro fertilization was performed in Irbil city/Iraq, according to private lab guidelines (Soran laboratory at Soran Private Hospital for Fertility Disease and Embryo Transfer).

Oocytes collection:

Clean the ovarian samples were dissecting from surroundings by clean sterilized scissors as same way as described in [7]. Before processing, samples were separated into two sets of 20 ovaries, with one oocyte collection process performed under aseptic circumstances in a Becker containing phosphate buffer saline (PBS) with an antibiotic solution for 5-10 minutes at room temperature (RT). It was carried out under aseptic settings in a well-sterilized hood cabinet to avoid air pollution or other polluted environments that could have influenced the final results. The following procedure was used to retrieve oocytes.

Ovarian slicing for oocytes collection:

Each ovarian sample was rinsed with distilled water and normal saline to complete clean it, before being suspended in a Becker containing PBS medium with antifungal and antibiotic treatments at RT. Each ovary was gripped with artery forceps slightly above the Becker solution preparation, then incised multiple times, primarily over the follicles, to include oocytes in the follicular fluid, and then soaked in Becker to ensure that all of the material was dumped down in the medium [8].

Oocytes evaluation:

During arrangement of cumulus cells surrounding oocytes and cytoplasm state, the approaches for collecting oocytes were subjected to quality control as described by [9]. The oocytes receive an excellent grade or grade A when they have numerous layers of cumulus cells and a translucent, homogenous, and uniform cytoplasm. The oocytes are given a fair or Grade B when they are less compact

cumulus cells that are transparent, less homogeneous (some granules may be present), and uniform. Oocytes with a mild or absent cumulus (denuded) and black, granular cytoplasm are classified as poor or grade C. After grading and quality assessing the collected oocytes, oocytes were transferred to another Petri-dish containing PBS medium as prepared, re-examining the dishes after these ova transports, and confirming that all selective ova were transported by aspiration with an automated micropipette. The ova were grading papers and keeping track of the numbers (Figure 1).

Oocytes maturation:

The maturation procedure was carried out on only good (grade A) oocytes, and the maturation medium was created according to lab protocols. The maturation media (MM) was equilibrated in a CO2 incubator for two hours before adding the oocytes; 5-6 ml of the earlier solution was placed in a glass petri dish, and the oocytes were added later. The matured oocytes were viewed under an inverted microscope, and the degree of maturation was measured as mentioned by [10]. The same maturation media was used to wash the graded and chosen oocytes many times, and the number of matured oocytes was tallied and recorded.

In vitro fertilization and embryo production:

The initial combination of the capacitated epididymal ram spermatozoa from slaughtered samples with the Petri dish containing the developed oocytes was handled and capacitated two hours before using heparin in a special petri dish. The diluted spermatozoa must yield $1\text{-}2*10^6$ spermatozoa [11]. The gametes were mixed and incubated. The samples were incubated for 28-30 hours at 5 % CO2 at 38.5°C and 90% relative humidity. The fertilization media included amino acid (arginine, methionine or alanine), LH, FSH, BSA, antibiotics, and antifungal preparations. Developed embryos must be inspected and monitored every 24 hours till 120 hours of incubation; embryos that show no signs of progress must be removed, and all developed embryos must be evaluated, with all results recorded.

Statistical analysis:

The results of the experiments were expressed as mean + percentage. ANOVA was used to compare distributed data (One-Way Analysis of Variance). Chi-squire Test was used to find significant variances between percentages. Sigma Stat was used to do the statistical analysis.

RESULTS

As presented in Table 1, there were a differences in the results obtained after adding the three amino acids, some groups recorded excellent results in the percentage of maturing and fertilization of eggs for the purpose of in vitro fertilization, while the alanine group was the least group. In the obtained results, the amino acid arginine (87.7%) recorded the highest fertilization rate obtained (20%) based on the evaluation of embryos when kept in a carbon dioxide incubator (CO2 incubator) after 120 hours of maturation and fertilization, followed by the amino acid methionine (85.5%) and control group where its results was (66.6%) with no significant changes ($p < 0.01$) between them , the significant changes recorded in Grade B embryos which higher in control group (33.3%) followed by methionine (14.3%.) and arginine (12.5%). With no significant record between them. While alanine group recorded just Grade C embryo with bad quality.

Table 1. The percentages of embryo development after adding arginine, methionine and alanine for 120 hours of maturation and fertilization of in vitro fertilization of Iraqi Awassi ewes.

Groups	**Total number of ova**	**Total number of fertilized eggs**	**Grade and percentage (%) of Embryonic development after 24-48 of division**					
			Grade A	**%**	**Grade B**	**%**	**Grade C**	**%**
Control	20	12	8.0	66.6[a]	4	33.3[a]	0.0	-----
Arginine	20	16	14.0	87.5[a]	2	12.5[b]	0.0	-----
methionine	20	14	12.0	85.7[a]	2	14.3[b]	0.0	-----
alanine	20	4	0.0	0.0[C]	0.0	0.0[C]	3	75
total	80	46	34	-------	9	------	-----	-----

*Different letters in same column refers to significant changes at $p< 0.01$.

The good maturation of the eggs after adding the amino acids and also control group reveled in Figures 1,2,3. While the state of degeneration or shrinkage regeneration and withdrawal of the contents of the cytoplasm to the center in the eggs showed in alanine group, figure 4. Arginine group (Figure 2) showed Maturation of the eggs after adding amino acid during incubation were examined by visual examination under inverted microscope revealed the best ovum quality (according to presence of intact zona pellucida and presence of first polar body in the margin of ova) were recorded with more numbers of eggs when compared with both Methionine and the control group (figures 2 & 3).

Alanine group reveled negative results, under inverted microscope and visual examination showed condition of regrowth or shrinkage and withdrawal of the cytoplasmic contents to the center in the eggs (Figure 4).

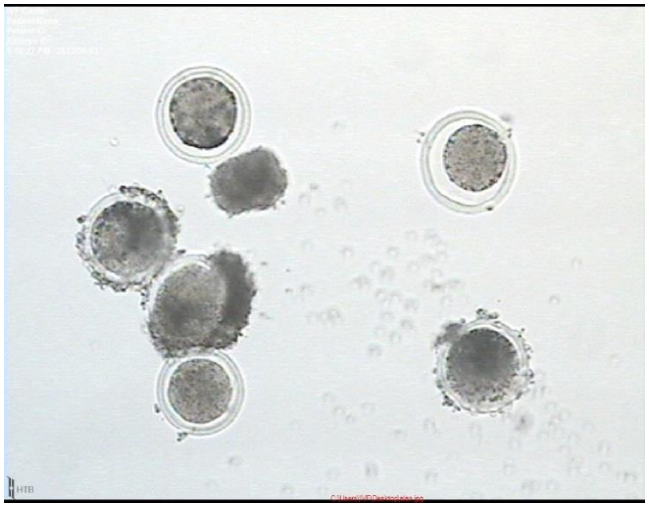

Figure 1. The process of maturation of eggs after incubating them in the methionine group under an inverted microscope, 10X, after 24 hours of incubation

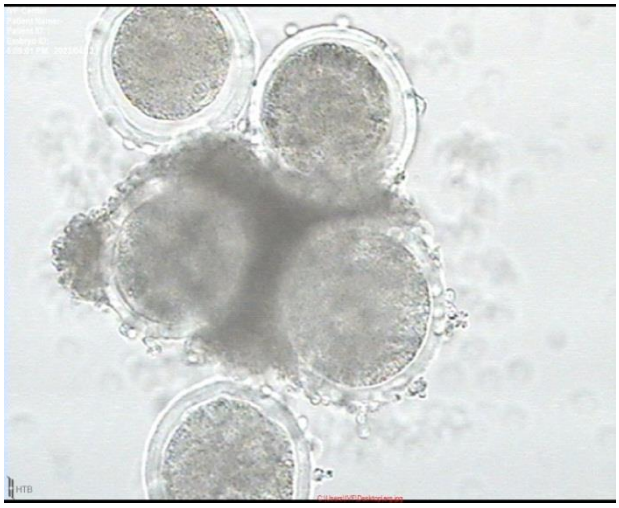

Figure 2. reveals the process of maturation of eggs after incubating them in the arginine group under an inverted microscope, 40X, after 24 hours of incubation

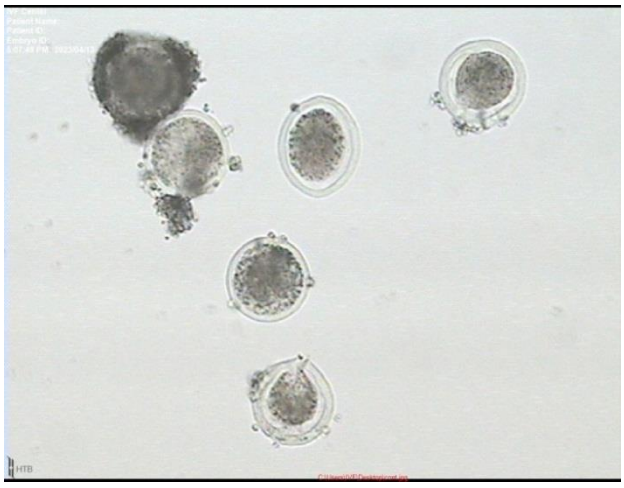

Figure 3. shows the process of maturation of eggs after incubating them in the control group under an inverted microscope, 10X, after 24 hours of incubation**.**

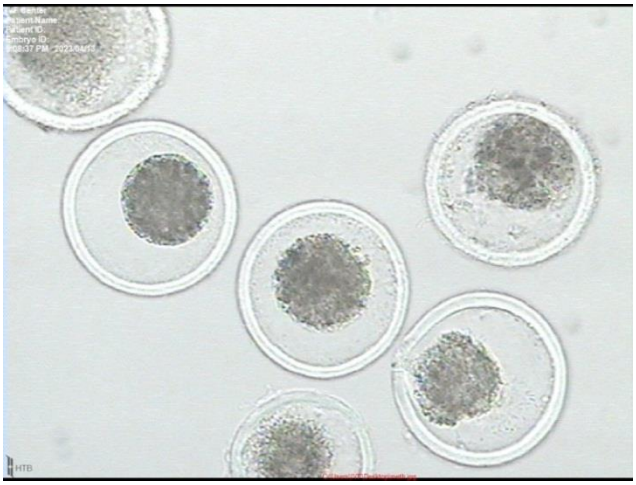

Figure 4. shows a state of shrinkage or regeneration and withdrawal of the contents of the cytoplasm to the center in the eggs of the alanine group under an inverted microscope, 40X, after 24 hours of incubation.

DISCUSSION

Embryo production using assisted reproductive technologies (ART), now considered a routine and successful treatment for infertility worldwide, has resulted in millions of live fetuses. Embryogenesis and divisions, especially in cases that suffer from repeated failure or during incubation, maturation of eggs, up to embryo transfer and implantation [12]. The addition of amino acids to the culture medium is beneficial to the embryo to varying extents in many species [13]. In a study conducted by [14] it was proved that amino acids, especially essential amino acids, are beneficial for the maturation of cytoplasmic eggs and early embryo development in the laboratory in a study they conducted on Holstein-Friesian cows in different proportions.

In the same way, the researcher indicated [15] that the addition of amino acids has a varying role, but in general it gives positive results if added with fixed specific ratios for its role at the level of metabolism that occurs in different stages of the development of the fertilized egg, leading to the formation of the blastoma cell blastocysts in a study conducted in Holstein Friesian cows.

The results of the present study indicate an improvement in the rates of maturation and fertilization in sheep eggs after adding the amino acid arginine at a concentration of (0.4 Mm), with good results compared to the rest of the amino acids used in the experiment, and this result is close to what the researcher [16] found in a study he conducted in cows, where he proved that the use of high concentrations of arginine (50 Mm) has a negative effect on the vitality and divisions of fertilized eggs, and that low concentrations of arginine (0-1Mm) have a positive effect on the development of embryos after fertilization, and attributed high concentration possibility leads to poisoning, whether to eggs or sperm, with this high level of arginine during fertilization and development of eggs, in addition to the effect on sperm as well. This agreement in the results of the current study with similar studies is due to the positive effect of arginine at the cell level and its being an important intermediate amino acid, not the production of many other substances, as arginine is one of the main precursors of nitric oxide (NO). Previous studies showed that the addition of L-arginine to IVFM was associated with an increase in the production of NO, nitric oxide, which in turn was associated with an improvement in spermatogenesis parameters, in addition to enhancing the ability to fertilize in many animals, including Rats [17]. Pigs [18], humans [19], buffaloes [20], and cattle [21,22-23], and we did not record a negative effect of arginine in affecting the fertilization processes or divisions of the egg.

The second amino acid, methionine, its effect was positive, but in the second place after arginine, according to the results of the present study. it was indicated that the effect of methionine begins at the level after the 4- cell stage, where its effect here is on the process of manufacturing proteins during embryonic cell divisions, and these proteins are important in the process of making genes for the embryo [6], and this may make methionine in second place after arginine due to time of action begin after arginine which were act immediately during maturation, fertilization and formation of gamete.

The results of the present study showed that the addition of alanine in high concentration (15Mm) led to a deterioration in the condition of the eggs after incubation, as it caused shrinkage or regeneration and the

withdrawal of the cytoplasmic contents to the medium. These results in agreed with what was indicated by the researcher [24] , the author concluded that treating pig eggs with low dose of alanine (0.363 Mm) during the maturation of eggs improves embryonic developmental efficiency after the fertilization process by increasing the content of glutathione inside the cells and increasing mRNA expression by Effect on the two genes POU5F1 and FGFR2, which are responsible for oocyte quality and embryonic development and he refers to The concentrations of alanine between (5-10 Mm) or more was negative in its effect on the level of cell division processes.

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CONCLUSION

It could be concluded from the current study that the arginine was the best of the three amino acids in stimulating divisions of the egg and improving the percentage of embryos produced in the laboratory after incubation for 120 hours.

CONFLICT OF INTEREST

There was no conflict of interest.

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